St. Xavier's College
(Autonomous), Mumbai

Department of Life Science and Biochemistry

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SLSC501PR

Internal Assessment Project Report

Isolation and identification of unknown protease producing bacteria from soil

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UID Number: 172007 **Date of Submission: 9-9-19**

INTRODUCTION:

Proteases refers to a group of enzymes whose catalytic function is to hydrolyse proteins. They are also called proteolytic enzymes or proteinases. Proteases are one of the most important group of industrial enzymes, and commercial proteases account for nearly 60% of the total industrial enzyme market. Bacillus proteases are predominantly extracellular and can be concentrated in the fermentation medium [2]. In the present study, Proteolytic enzyme bacteria were derived from Home made compost which made up of rotten vegetables. The main aim of this project was to isolate the pure culture of single proteolytic enzyme bacteria and identify the species.

Materials and Methods:

Composition of Nutrient agar plate:

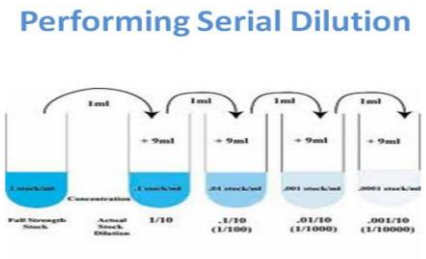
0.5% Peptone - this provides organic nitrogen. 0.3% beef extract/yeast extract - the water-soluble content of these contribute vitamins, carbohydrates, nitrogen, and salts. 1.5% agar - this gives the mixture solidity.

Composition of Skim milk agar plate:

52.15% lactose, 38.71% protein (31.18% casein, 7.53% whey protein), 1.08% fat, and 8.06% ash.

Serial dilution [1]: [Pipette, test tubes, cannister, vortex]

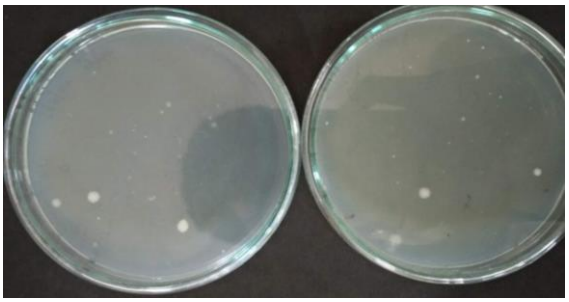
- With a sterile 10ml pipette aseptically transfer 9ml of saline to 5 sterile test tubes.
- Transfer aseptically 1g of the given compost to the first dilution tube. Vortex it. This is a 10^{-1} dilution.
- Using a fresh sterile 1ml pipette to transfer 1ml culture from the 10^{-1} dilution to the next test tube. This is a 10^{-2} dilution.
- Continue to serially dilute the culture up to 10^{-5} dilution.



- Using a fresh sterile 1ml pipette, aspirate 0.1ml from the 10^{-5} dilution and add to a sterile nutrient agar plate and skim milk agar plate too.
- Sterilize the spreader by flame sterilization using alcohol. Let the spreader cool and aseptically surface spread the culture using a sterile spreader till the surface of nutrient agar plate and skim milk agar plate are dry.
- Repeat the steps for 10^{-4} and 10^{-3} dilutions.
- Incubate the plates in inverted position in the 37^{0}c incubator overnight.

After the incubation, as the plates were:

Skim milk agar plate

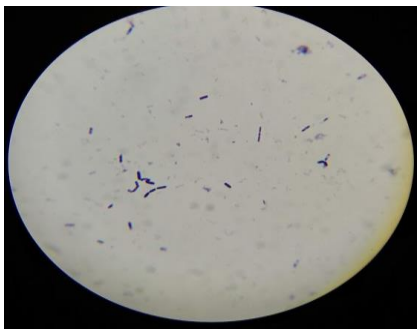


Nutrient agar plate

Gram stain [1]: [Gram's crystal violet and iodine, Acetone-alcohol, Safranin, Clean glass slide, nichrome loop, cedar-wood oil, compound microscope]

- Prepare a thin smear of the culture on a clean glass slide.
- Allow the smear to air dry and then heat fix the smear by passing it through the flame. Cool the slide to room temperature.
- Flood the smear with crystal violet solution for 1 minute.
- The slide is gently rinsed in clean tap water.
- Flood the smear with gram's iodine mordant and let stand for 1 minute.
- Decolorize with acetone alcohol. Add the reagent drop by drop until the alcohol runs almost clear.
- The slide is then gently rinsed in clean tap water.
- Counter stain with safranin for one and half minutes.
- The slide is then gently rinsed in clean tap water.
- Air-dry the slide.
- Put a drop of cedar-wood oil and observe under 100x oil immersion lens.

Gram staining was performed and it's gave:



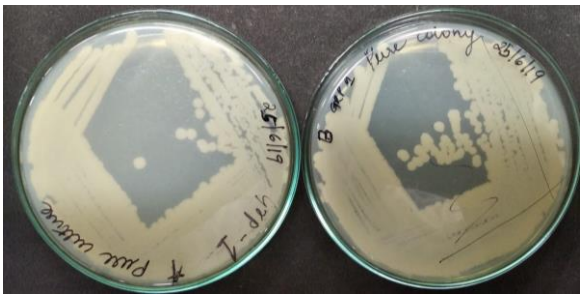
Color	violet
Gram nature	positive
Morphology	bacilli
Occurrence	chain

Composition of Slant:

Agar, a pH-sensitive dye (phenol red), 1% lactose, 1% sucrose, 0.1% glucose, and sodium thiosulfate and ferrous sulphate or ferrous ammonium sulphate.

Need to do Gram's staining regular basis to track on the microorganism. A good one colony was picked from Nutrient agar plate and skim milk agar plate. It was again spread into the plate by hexagonal method and also made the slant for culturing the organism.

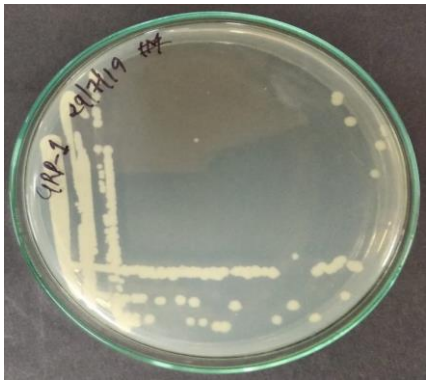
Slant



Hexagonal method

Nichrome loop: It usually made of platinum or nichrome wire in which the tip forms a small loop with a diameter of about 5 mm, and is used to smear, streak, or take an inoculum from, a culture of microorganisms.

A colony was picked from the plate which was performed by hexagonal method and again did hexagonal method in the plate with nichrome loop to get a pure individual colony.



Hexagonal method

Isolation of genomic DNA [1]:

- Add super optimal broth media to overnight culture of compost and mix it properly.
- Take 1.5ml culture in Eppendorf tube and spin it at 14000rpm/5min
- In the pellet add tris EDTA buffer (400 micro litre) and 10%SDS (100 micro litre) and mix it.
- Keep the tube in incubator at 60^0c for 5-10min.

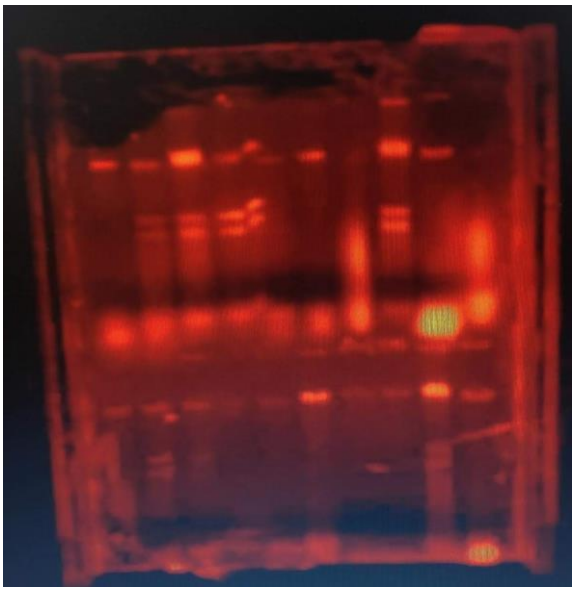
- After incubation, add 6M sodium per chlorate (100 micro litre) to the tube. Again, Keep the tube in incubator at 60^0c for 5-10min.
- Add equal volume of chloroform (600 micro litre).
- Centrifuge at 14000rpm/20min
- Get the three layers formed in the tube (aqueous, organic and pellet).
- Discard the supernatant and rinse the DNA pellet with 1ml of 70% alcohol
- Now again spin at maximum speed.
- Check genomic DNA on agarose gel.

Why gel electrophoresis is used for?

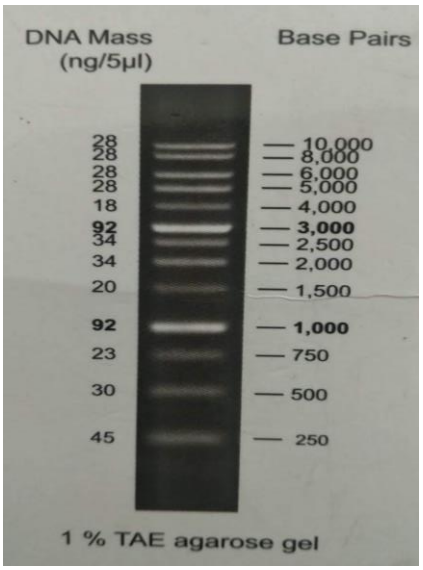
Gel electrophoresis [4] is a technique used to separate DNA fragments, other macromolecules, such as RNA and protein based on their size and charge. Based on their size and charge, the molecules will travel through the gel in different directions or at different speeds, allowing them to be separated from one another. In gel electrophoresis, negatively charged DNA fragments travel through the porous gel towards the positive end of the tray when a charge is applied to the gel. Large fragments do not travel as far as small fragments. We can know the exact order of bases in the length of DNA.

Agarose gel electrophoresis [1]:

- Prepare a .8% agarose gel in 1x TBE buffer. Boil till agarose dissolves completely and a clear solution result.
- Meanwhile, gel the casting tray with cello tape and place the comb such that it is 2cm away from the cathode.
- Allow it to cool for a little while time and then pipette about 10-15micro litre of the ethidium bromide stock solution in it and gently swirl it to mix it.
- Pour the agarose solution in the central part of the tank. Do not generate air bubbles. The thickness of the gel should be around 0.5 to 0.9cm. keep the gel undisturbed at room temperature for the agarose to solidify.
- Pour 1X TBE buffer into the gel tank till the buffer level stands at 0.5 to 0.8cm above the gel surface. Gently lift the comb, ensuring that wells remain intact.
- Load 2 micro liter of dye, 7 micro liter of genomic DNA and also add 10 micro liter of Ethidium bromide which is fluoresces under UV.
- Set it at maximum current and a constant voltage of about 60-75Volts. Let it run till the tracking dye from the well reaches of the gel
- View the gel on a UV trans-illuminator.



Genomic DNA in UV trans illuminator



Flow chart scale

According to the flow chart, our genomic DNA molecular weight may be 750 to 1000 kilo Daltons or 750 to 1000 base pair.

Polymerase chain reaction [6,5]: [forward and reverse primer, Taq master mix, Water for injection]

PCR is a method used to make several copies of a specific DNA segment. Using PCR, copies of DNA sequences are exponentially amplified to generate thousands to millions of more copies of that particular DNA segment.

The basic steps are:

Denaturation (95^0C)˙: Heat the reaction strongly to separate, or denature, the DNA strands. This provides single-stranded template for the next step.

Annealing(55^0C): Cool the reaction so the primers can bind to their complementary sequences on the single-stranded template DNA.

Extension (72°C): Raise the reaction temperatures so *Taq* polymerase extends the primers, synthesizing new strands of DNA.

The important points of PCR are:

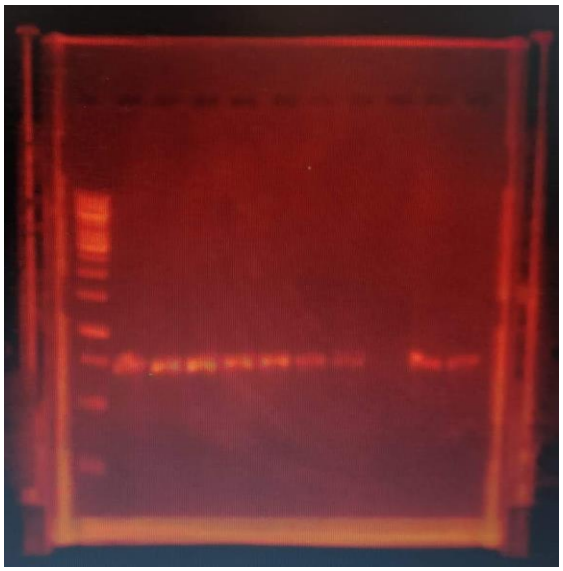
- Primers should not be more than 30 nucleotides and primer always 5˙-3˙ direction.
- PCR doesn't work when excess of genomic DNA.
- Separation can only happen at high temperatures.

We need to find the temperature of annealing also based on the sequences.

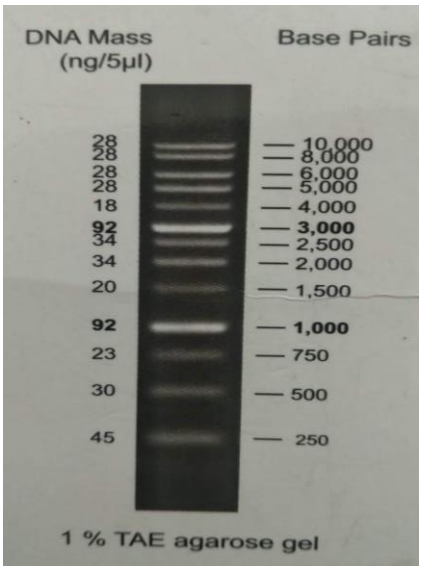
- Temperature of melting forward primer = 2*(A+T) + 4*(G+C)
- Temperature of melting reverse primer = 2*(A+T) + 4*(G+C)

Procedure:

- Add 1 micro liter of 16srRNA, 1 micro liter of forward primer, 1 micro liter of reverse primer, 1 micro liter of Taq master mix and 1 micro liter of water for injection into the Eppendorf tube.
- Keep the tube into the PCR for 2 hours that's what we did. It can change depends on the models of PCR.
- After 2 hours, remove the tube and Check genomic DNA on agarose gel.
- As the procedure of gel electrophoresis mentioned earlier. Repeat again.
- View the gel on a UV trans-illuminator.



Genomic DNA in UV trans illuminator



Flow chart scale

According to the flow chart, our genomic DNA molecular weight may be 750 to 1000 kilo Daltons or 750 to 1000 base pair.

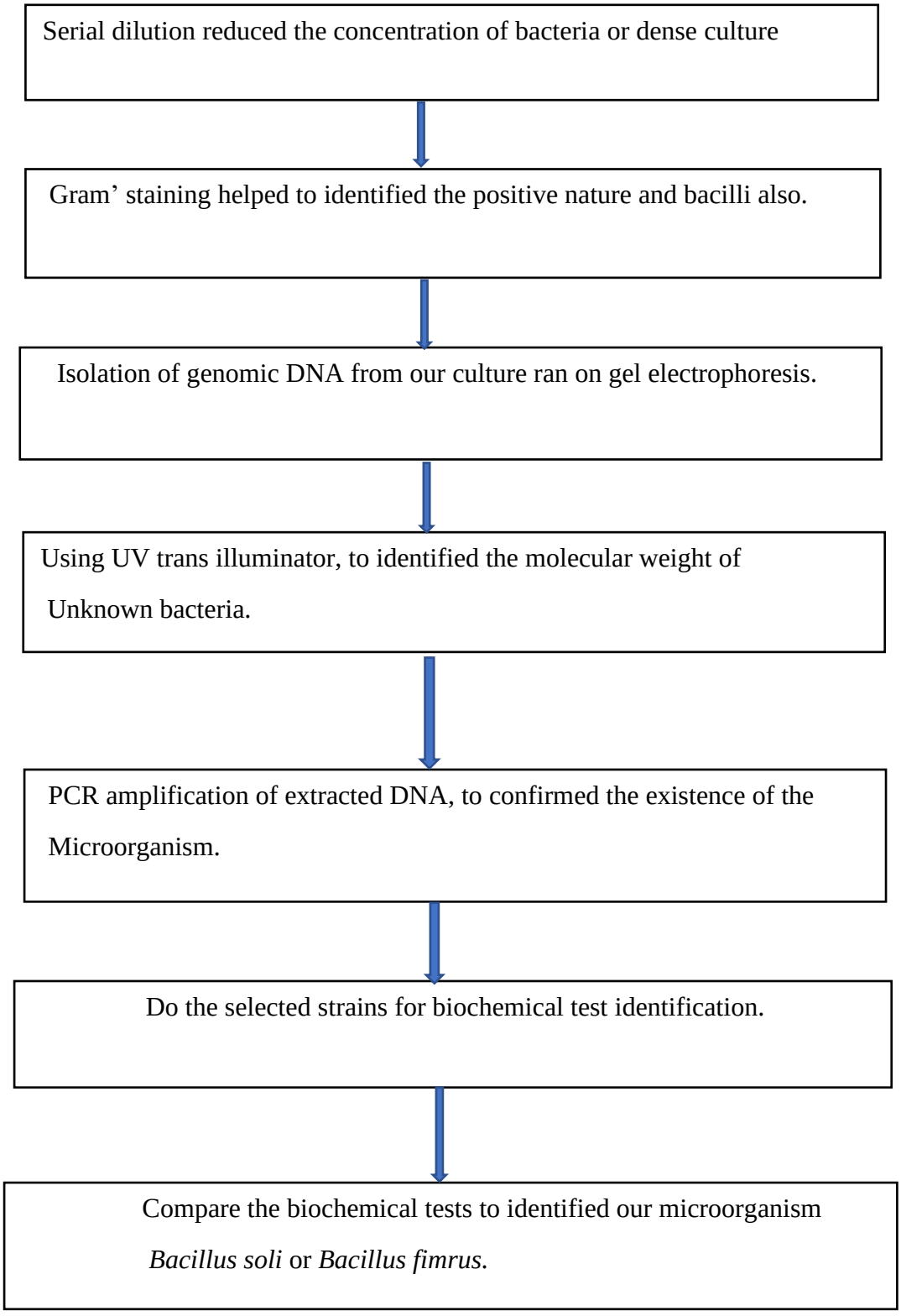
Biochemical tests:

Tests	Results
glucose	Positive
Sucrose	Positive
Mannitol	Negative
Galactose	Negative
Urea	Positive
Lactose	Negative
Xylulose	Negative
7% Nacl	Positive
Nitrate	Positive
Glucose 6 Po4	Positive
Catalase	Negative
Maltose	Positive

Tests	Results
Indole	Negative
Methyl red	Positive
Voges Proskaeur	Negative
Citrate utilization	Negative
Sugar+ Gas	Negative

From the Biochemical tests, we concluded our micro organism may be *Bacillus soli* or *Bacillus fimrus.*

Results [8,7]:



Bacillus	Casein	Urea	Citrate	7%Nacl	V.P	Sucrose	Maltose	Nitrate	Indole	Galactose	Lactose
Firmus	+	-	-	+	-	-	+	+	-		-
Decolorationis	+	-	-	+	-			+	-		-
Novalis	+	-	-	-	-			+	-		-
psychrosaccharolyticus	+	+	-	-	-			+	-		-
Siralis	+	-	-	+	-		-	+	-	-	
Soli	+	-	-	-	-			+			-
Vireti	+	+	-	+	-	+	+	+			-
Galactosidilyticus	+	-	-	-	-			+	-		-

Compare these results and what we got from biochemical test for our culture, we concluded the microorganism may be *Bacillus soli* or *Bacillus fimrus.*

Discussion:

It is an excellent method to learn the experiments from a project. Before this project, I don't know much information about the PCR technique, gel electrophoresis, and UV transilluminator. Now, I get to know so much information about these techniques and instruments. I had a good experience when I am doing this. In my point of view, Gram's staining and biochemical tests play an important role to identify the microorganism. It's very interest to find the particular microorganism from the soil. This project will be going to help in my future definitely for further analysis of microorganism.

Reference:

1. Genetics 1nd and 2nd year lab journal.
2. www.sciencelearn.org.nz
3. Wps.prenhail.com
4. www.khanacademy.org
5. www.promega.in
6. https://study.com
7. DR.A.C DEB,1996[practical biochemistry]
8. Bergey's manual

Roll No:125 UID: 172007

Date of submission:9-9-19 Total Marks obtained: _____/20

Grading Rubric:

	GRADED ITEM	Max. Score	Obtained Score
1.	Format Guidelines followed? (margins, font, spacing, title pg, etc.), Neatness, spelling, grammar, nomenclature • Report written in professional, objective manner--no personal pronouns • Have all scientific names been written using proper nomenclature? • Is grammar and spelling proper and accurate? Is report prepared in a tidy fashion according to requirements?	**2**	
2.	Introduction Purpose of study clearly stated	**2**	
3.	Materials and Methods • Was each technique used described in appropriate detail? • Was streak plate isolation described and any problems discussed? • Were isolation and biochemical testing procedures performed correctly? • Was isolation accomplished and pure cultures maintained regularly.	**4**	
4.	Results Tables - • Flow Chart: Is path of action indicated for the unknown, including any extra work? • Is there a complete table of test results showing visual AND interpretive information? • Were results correctly interpreted (info in notebook and in discussion section)	**4**	
5.	Correct ID • Was the unknown correctly identified? • If unknown ID incorrect, was it the result of student error (technique or judgement) or a factor out of the student's control?	**1**	
6.	Discussion • Has student avoided repetition of methods and results in this section? • Have rationales for identifications been adequately described? • Has student demonstrated he/she can make appropriate conclusions? • Has students identified any errors in technique or judgment and suggested alternatives for future work? • Is discussion written in a professional manner? • Have all changes in original plan been discussed?	**3**	
7.	References • Have all required components been included in proper scientific format? • Have all authors been cited, in the order in which they appear?	**1**	
9.	Laboratory working and discussions • Participation in discussions, following proper techniques, following up with results and suggesting logical steps for further tests.	**3**	
10.	Report submitted late (-10% per day)		
	TOTAL	**20**	

Teacher-in-charge